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(54) Title: EXPRESSION OF UROKINASE PLASMINOGEN ACTIVATOR INHIBITORS

(57) Abstract

A method for preparing a urokinase-type plasminogen activator inhibitor by expressing $HuPA_{1-48}$ from yeast is disclosed.

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Expression of Urokinase Plasminogen Activator Inhibitors

Description

Technical Field

This invention relates to the fields of cellular biology and protein expression. More particularly, the invention relates to peptide ligands of the urokinase plasminogen activator receptor, and methods for preparing the same.

Background of the Invention

Urokinase-type plasminogen activator (uPA) is a multidomain serine protease, having a catalytic "B" chain (amino acids 144-411), and an amino-terminal fragment ("ATF", aa 1-143) consisting of a growth factor-like domain (4-43) and a kringle (aa 47-135). The uPA kringle appears to bind heparin, but not fibrin, lysine, or aminohexanoic acid. The growth factor-like domain bears some similarity to the structure of epidermal growth factor (EGF), and is thus also referred to as an "EGF-like" domain. The single chain pro-uPA is activated by plasmin, cleaving the chain into the two chain active form, which is linked together by a disulfide bond.

uPA binds to its specific cell surface receptor (uPAR). The binding interaction is apparently mediated by the EGF-like domain (S.A. Rabbani *et al.*, J Biol Chem (1992) 267:14151-56). Cleavage of pro-uPA into active uPA is accelerated when pro-uPA and plasminogen are receptor-bound. Thus, plasmin activates pro-uPA, which in turn activates more plasmin by cleaving plasminogen. This positive feedback cycle is apparently limited to the receptor-based proteolysis on the cell surface, since a large excess of protease inhibitors is found in plasma, including α_2 antiplasmin, PAI-1 and PAI-2.

Plasmin can activate or degrade extracellular proteins such as fibrinogen, fibronectin, and zymogens. Plasminogen activators thus can regulate extracellular proteolysis, fibrin clot lysis, tissue remodeling, developmental cell migration, inflammation, and metastasis. Accordingly, there is great interest in developing uPA inhibitors and uPA receptor antagonists. E. Appella *et al.*, J Biol Chem (1987) 262:4437-40 determined that receptor binding activity is localized in the EGF-like domain, and that residues 12-32 appear to be critical for binding. The critical domain alone (uPA_{12-32}) bound uPAR with an affinity of 40 nM (about 100 fold less than intact ATF).

S.A. Rabbani *et al.*, *supra*, disclosed that the EGF-like domain is fucosylated at Thr_{18}, and reported that fucosylated EGF-like domain (uPA_{4-43}, produced by cleavage from pro-uPA) was mitogenic for an osteosarcoma cell line, SaOS-2. In contrast, non-fucosylated EGF-like domain bound uPAR with an affinity equal to the fucosylated EGF-like domain, but exhibited no mitogenic activity. Non-fucosylated EGF-like domain competed for binding to uPAR with fucosylated EGF-like domain, and reduced the mitogenic activity observed. Neither EGF-like domain was mitogenic in U937 fibroblast cells.

Previously, it was suggested that an "epitope library" might be made by cloning synthetic DNA that encodes random peptides into filamentous phage vectors (Parmley and Smith, Gene (1988) 73:305). It was proposed that the synthetic DNA be cloned into the coat protein gene III because of the likelihood of the encoded peptide becoming part of pIII without significantly interfering with pIII's function. It is known that the amino terminal half of pIII binds to the F pilus during infection of the phage into *E. coli*. It was suggested that such phage that carry and express random peptides on their cell surface as part of pIII may provide a way of identifying the epitopes recognized by antibodies, particularly using antibody to affect the purification of phage from the library. Devlin, PCT WO91/18980 (incorporated herein by reference) described a method for producing a library consisting of random peptide sequences presented on filamentous

phage. The library can be used for many purposes, including identifying and selecting peptides that have a particular bioactivity. An example of a ligand binding molecule would be a soluble or insoluble cellular receptor (*i.e.*, a membrane bound receptor), but would extend to virtually any molecule, including enzymes, that have the sought after binding activity. Description of a similar library is found in Dower *et al.*, WO91/19818. The present invention provides a method for screening such libraries (and other libraries of peptides) to determine bioactive peptides or compounds. Kang *et al.*, WO92/18619 disclosed a phage library prepared by inserting into the pVIII gene.

However, both the pIII and pVIII proteins are expressed in multiple copies in filamentous bacteriophage. As a result, the phage are selected and amplified based on their avidity for the target, rather than their affinity. To overcome this problem, a method for monovalent (only one test peptide per phage) phage display has been developed (H.B. Lowman *et al.*, Biochem (1991) 30:10832-38). To obtain monovalent display, the bacterial host is coinfected with the phage library and a large excess of "helper" phage, which express only wild-type pIII (and/or pVIII) and are inefficiently packaged. By adjusting the ratio of display phage to helper phage, one can adjust the ratio of modified to wild-type display proteins so that most phage have only one modified protein. However, this results in a large amount of phage having only wild-type pIII (or pVIII), which significantly raises the background noise of the screening.

Disclosure of the Invention

One aspect of the invention is a method for producing non-fucosylated uPA EGF-like domain, particularly uPA_{1-48}.

Another aspect of the invention is non-fucosylated uPA_{1-48}, which is useful for inhibiting the mitogenic activity of uPA in cancer cells.

Another aspect of the invention is a method for treating cancer and metastasis by administering an effective amount of a non-fucosylated uPA EGF-like domain, particularly uPA_{1-48}.

Another aspect of the invention is a method for pre-enriching a monovalent phage display mixture prior to screening for binding to a target, by providing a mixture of monovalent display phage and non-displaying phage, wherein the monovalent display phage display both a candidate peptide and a common peptide, the common peptide is identical for each monovalent display phage, and the candidate peptide is different for different monovalent display phage; and separating all phage displaying the common peptide from phage not displaying a common peptide.

Modes of Carrying Out The Invention

A. Definitions

The term "huPA" refers specifically to human urokinase-type plasminogen activator. The "EGF-like domain" is that portion of the huPA molecule responsible for mediating huPA binding to its receptor (uPAR). The EGF-like domain, sometimes called the growth factor-like domain ("GFD"), is located within the first 48 residues of huPA. The critical residues (essential for binding activity) have been localized to positions 12-32, although a peptide containing only those residues does not exhibit a binding affinity high enough to serve as a useful receptor antagonist.

The term "huPAR antagonist polypeptide" refers to a polypeptide having a sequence identical to the EGF-like domain of huPA (residues 1-48), or an active portion thereof. An "active portion" is one which lacks up to 10 amino acids, from the N-terminal or C-terminal ends, or a combination thereof, of the $huPA_{1-48}$ polypeptide, and exhibits a $K_d \leq 5$ nM with huPAR. The term "active analog" refers to a polypeptide differing from the sequence of the EGF-like domain of $huPA_{1-48}$ or an active portion thereof by 1-7 amino acids, but which

still exhibits a $K_d \leq 5$ nM with huPAR. The differences are preferably conservative amino acid substitutions, in which an amino acid is replaced with another naturally-occurring amino acid of similar character. For example, the following substitutions are considered "conservative": Gly ⇔ Ala; Val ⇔ Ile ⇔ Leu; Asp ⇔ Glu; Lys ⇔ Arg; Asn ⇔ Gln; and Phe ⇔ Trp ⇔ Tyr. Nonconservative changes are generally substitutions of one of the above amino acids with an amino acid from a different group (*e.g.*, substituting Asn for Glu), or substituting Cys, Met, His, or Pro for any of the above amino acids. The huPAR antagonist polypeptides of the invention should be substantially free of peptides derived from other portions of the huPA protein. For example, a huPAR antagonist composition should contain less than 20 wt% uPA B domain (dry weight, absent excipients), preferably less than 10 wt% uPA-B, more preferably less than 5 wt% uPA-B, most preferably no detectable amount. The huPAR antagonist polypeptides also preferably exclude the kringle region of uPA.

The term "expression vector" refers to an oligonucleotide which encodes the huPAR antagonist polypeptide of the invention and provides the sequences necessary for its expression in the selected host cell. Expression vectors will generally include a transcriptional promoter and terminator, or will provide for incorporation adjacent to an endogenous promoter. Expression vectors will usually be plasmids, further comprising an origin of replication and one or more selectable markers. However, expression vectors may alternatively be viral recombinants designed to infect the host, or integrating vectors designed to integrate at a preferred site within the host's genome. Expression vectors may further comprise an oligonucleotide encoding a signal leader polypeptide. When "operatively connected", the huPAR antagonist is expressed downstream and in frame with the signal leader, which then provides for secretion of the huPAR antagonist polypeptide by the host to the extracellular medium. Presently preferred signal leaders are the *Saccharomyces cerevisiae* α-factor leader (partic-

ularly when modified to delete extraneous Glu-Ala sequences), and the ubiquitin leader (for intracellular expression).

The term "transcriptional promoter" refers to an oligonucleotide sequence which provides for regulation of the DNA → mRNA transcription process, typically based on temperature, or the presence or absence of metabolites, inhibitors, or inducers. Transcriptional promoters may be regulated (inducible/repressible) or constitutive. Yeast glycolytic enzyme promoters are capable of driving the transcription and expression of heterologous proteins to high levels, and are particularly preferred. The presently preferred promoter is the hybrid ADH2/GAP promoter described in Tekamp-Olson *et al.*, US 4,876,197 (incorporated herein by reference), comprising the *S. cerevisiae* glyceraldehyde-3-phosphate dehydrogenase promoter in combination with the *S. cerevisiae* alcohol dehydrogenase II upstream activation site.

The term "host" refers to a yeast cell suitable for expressing heterologous polypeptides. There are a variety of suitable genera, such as *Saccharomyces, Schizosaccharomyces, Kluveromyces, Pichia, Hansenula*, and the like. Presently preferred are yeast of the *Saccharomyces* genus, particularly *Saccharomyces cerevisiae*.

The term "uPA-mediated disorder" refers to a disease state or malady which is caused or exacerbated by a biological activity of uPA. The primary biological activity exhibited is plasminogen activation. Disorders mediated by plasminogen activation include, without limitation, inappropriate angiogenesis (*e.g.*, diabetic retinopathy, corneal angiogenesis, Kaposi's sarcoma, and the like), metastasis and invasion by tumor cells, and chronic inflammation (*e.g.*, rheumatoid arthritis, emphysema, and the like). Fucosylated ATF is also mitogenic for some tumor cells (*e.g.*, SaOS-2 osteosarcoma cells), which sometimes self-activate in an autocrine mechanism. Accordingly, the huPAR antagonist of the invention is effective in inhibiting the proliferation of uPA-activated tumor cells.

The term "effective amount" refers to an amount of huPAR antagonist polypeptide sufficient to exhibit a detectable therapeutic effect. The therapeutic effect may include, for example, without limitation, inhibiting the growth of undesired tissue or malignant cells, inhibiting inappropriate angiogenesis, limiting tissue damage caused by chronic inflammation, and the like. The precise effective amount for a subject will depend upon the subject's size and health, the nature and severity of the condition to be treated, and the like. Thus, it is not possible to specify an exact effective amount in advance. However, the effective amount for a given situation can be determined by routine experimentation based on the information provided herein.

The term "pharmaceutically acceptable" refers to compounds and compositions which may be administered to mammals without undue toxicity. Exemplary pharmaceutically acceptable salts include mineral acid salts such as hydrochlorides, hydrobromides, phosphates, sulfates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates, and the like.

The term "pre-enriching" refers to increasing the concentration of candidate phage in a monovalent phage display mixture by removing phage which do not have a candidate peptide. A "monovalent phage display mixture" is a mixture of phage containing recombinant phage and helper phage in a ratio such that most phage display at most one recombinant surface protein.

The term "common peptide" refers to a distinctive heterologous (not wild-type) peptide sequence which is displayed identically by all recombinant members of a phage (or other host) library. The common peptide is preferably an epitope recognized by a high-affinity antibody, which is not cross-reactive with any epitopes naturally occurring in the wild-type phage. The common peptide permits one to select all recombinant phage (having a common peptide and a random candidate peptide) as a set, and purify them away from non-recombinant (wild-type) phage. The presently preferred common peptide is Glu-Tyr-Met-Pro-Met-Glu.

B. General Method

The present invention relies on the fact that yeast do not fucosylate proteins upon expression, but are able to express properly folded, active uPA and fragments. One may employ other eukaryotic hosts in the practice of the invention as long as the host is incapable of fucosylating proteins, whether naturally or due to manipulation (*e.g.*, genetic mutation or antibiotic treatment). Presently preferred hosts are yeasts, particularly *Saccharomyces*, *Schizosaccharomyces*, *Kluveromyces*, *Pichia*, *Hansenula*, and the like, especially *S. cerevisiae*. Strains AB110 and MB2-1 are presently preferred.

The expression vector is constructed according to known methods, and typically comprises a plasmid functional in the selected host. The uPA sequence used may be cloned following the method described in Example 1 below. Variations thereof (*i.e.*, active fragments and active analogs) may be generated by site-specific mutagenesis, imperfect PCR, and other methods known in the art. Stable plasmids generally require an origin of replication (such as the yeast 2μ ori), and one or more selectable markers (such as antibiotic resistance) which can be used to screen for transformants and force retention of the plasmid. The vector should provide a promoter which is functional in the selected host cell, preferably a promoter derived from yeast glycolytic enzyme promoters such as GAPDH, GAL, and ADH2. These promoters are highly efficient, and can be used to drive expression of heterologous proteins up to about 10% of the host cell weight. The presently preferred promoter is a hybrid ADH2/GAP promoter comprising the *S. cerevisiae* glyceraldehyde-3-phosphate dehydrogenase promoter in combination with the *S. cerevisiae* alcohol dehydrogenase II upstream activation site.

The expression vector should ideally provide a signal leader sequence between the promoter and the huPAR antagonist polypeptide sequence. The signal leader sequence provides for translocation of the huPAR antagonist polypeptide through the endoplasmic reticulum and export from the cell into the

extracellular medium, where it may be easily harvested. There are a number of signal leader sequences known that are functional in yeast. The yeast α-factor leader is presently preferred (see U.S. 4,751,180, incorporated herein by reference).

Alternatively, the vector may provide for integration into the host genome, as is described by Shuster, PCT WO92/01800, incorporated herein by reference.

Transformations into yeast can be carried out according to the method of A. Hinnen *et al.*, Proc Natl Acad Sci USA (1978) 75:1929-33, or H. Ito *et al.*, J Bacteriol (1983) 153:163-68. After DNA is taken up by the host cell, the vector integrates into the yeast genome at one or more sites homologous to its targeting sequence. It is presently preferred to linearize the vector by cleaving it within the targeting sequence using a restriction endonuclease, as this procedure increases the efficiency of integration.

Following successful transformations, the number of integrated sequences may be increased by classical genetic techniques. As the individual cell clones can carry integrated vectors at different locations, a genetic cross between two appropriate strains followed by sporulation and recovery of segregants can result in a new yeast strain having the integrated sequences of both original parent strains. Continued cycles of this method with other integratively transformed strains can be used to further increase the copies of integrated plasmids in a yeast host strain. One may also amplify the integrated sequences by standard techniques, for example by treating the cells with increasing concentrations of copper ions (where a gene for copper resistance has been included in the integrating vector).

Correct ligations for plasmid construction may be confirmed by first transforming *E. coli* strain MM294 obtained from *E. coli* Genetic Stock Center, CGSC #6135, or other suitable host with the ligation mixture. Successful transformants are selected by ampicillin, tetracycline or other antibiotic resistance or

using other markers depending on the plasmid construction, as is understood in the art. Plasmids from the transformants are then prepared according to the method of D.B. Clewell *et al.*, Proc Natl Acad Sci USA (1969) 62:1159, optionally following chloramphenicol amplification (D.B. Clewell, J Bacteriol (1972) 110:667). Isolated DNA is analyzed by restriction mapping and/or sequenced by the dideoxy method of F. Sanger *et al.*, Proc Natl Acad Sci USA (1977) 74:5463 as further described by Messing *et al.*, Nucl Acids Res (1981) 9:309, or by the method of Maxam and Gilbert, Meth Enzymol (1980) 65:499.

huPAR antagonist polypeptides may be assayed for activity by methods known in the art. For example, one may assay competition of the antagonist against native uPA for cell surface receptor binding. Competition for the receptor correlates with inhibition of uPA biological activity. One may assay huPAR antagonist polypeptides for anti-mitogenic activity on appropriate tumor cell lines, such as the osteosarcoma cell line SaOS-2 described in the art. Inhibition of mitogenic activity may be determined by comparing the uptake of ^{3}H-T in osteosarcoma cells treated with the antagonist against controls. One may also assay huPAR antagonists for anti-invasive activity on appropriate tumor cell lines, such as HOC-1 and HCT116 (W. Schlechte *et al.*, Cancer Comm (1990) 2:173-79; H. Kobayashi *et al.*, Brit J Cancer (1993) 67:537-44).

huPAR antagonists are administered orally, topically, or by parenteral means, including subcutaneous and intramuscular injection, implantation of sustained release depots, intravenous injection, intranasal administration, and the like. When used to treat tumors, it may be advantageous to apply the huPAR antagonist directly to the site, *e.g.*, during surgery to remove the bulk of the tumor. Accordingly, huPAR antagonist may be administered as a pharmaceutical composition comprising huPAR antagonist in combination with a pharmaceutically acceptable excipient. Such compositions may be aqueous solutions, emulsions, creams, ointments, suspensions, gels, liposomal suspensions, and the like. Suitable excipients include water, saline, Ringer's solution, dextrose solution, and

solutions of ethanol, glucose, sucrose, dextran, mannose, mannitol, sorbitol, polyethylene glycol (PEG), phosphate, acetate, gelatin, collagen, Carbopol®, vegetable oils, and the like. One may additionally include suitable preservatives, stabilizers, antioxidants, antimicrobials, and buffering agents, for example, BHA, BHT, citric acid, ascorbic acid, tetracycline, and the like. Cream or ointment bases useful in formulation include lanolin, Silvadene® (Marion), Aquaphor® (Duke Laboratories), and the like. Other topical formulations include aerosols, bandages, and other wound dressings. Alternatively, one may incorporate or encapsulate the huPAR antagonist in a suitable polymer matrix or membrane, thus providing a sustained-release delivery device suitable for implantation near the site to be treated locally. Other devices include indwelling catheters and devices such as the Alzet® minipump. Ophthalmic preparations may be formulated using commercially available vehicles such as Sorbi-care® (Allergan), Neodecadron® (Merck, Sharp & Dohme), Lacrilube®, and the like, or may employ topical preparations such as that described in US 5,124,155, incorporated herein by reference. Further, one may provide a huPAR antagonist in solid form, especially as a lyophilized powder. Lyophilized formulations typically contain stabilizing and bulking agents, for example human serum albumin, sucrose, mannitol, and the like. A thorough discussion of pharmaceutically acceptable excipients is available in Remington's Pharmaceutical Sciences (Mack Pub. Co.).

The amount of huPAR antagonist required to treat any particular disorder will of course vary depending upon the nature and severity of the disorder, the age and condition of the subject, and other factors readily determined by one of ordinary skill in the art. The appropriate dosage may be determined by one of ordinary skill by following the methods set forth below in the examples. As a general guide, about 0.01 mg/Kg to about 50 mg/Kg huPAR antagonist administered i.v. or subcutaneously is effective for inhibiting tissue damage due to chronic inflammation. For treating corneal angiogenesis, huPAR

antagonist may be administered locally in a gel or matrix at a concentration of about 0.001 mg/Kg to about 5 mg/Kg.

C. Examples

The examples presented below are provided as a further guide to the practitioner of ordinary skill in the art, and are not to be construed as limiting the invention in any way.

Example 1

(Cloning and Expression of $huPA_{1-48}$)

DNA encoding residues 1-48 of mature human uPA (huPA) was cloned into a yeast expression vector as a fusion with the yeast alpha-factor leader (αFl), under transcriptional control of a hybrid ADH2-GAP promoter. The αFl is described in Brake, US 4,870,008, incorporated herein by reference. The hybrid ADH2-GAP promoter is described in Tekamp-Olson *et al.*, US 4,876,197, and Tekamp-Olson *et al.*, US 4,880,734, both incorporated herein by reference.

The gene encoding huPA was obtained by PCR using the following sense and nonsense primers:

5'-ATGCTAGATCTAATGAACTTCATCAGGTACCATCG-3' (SEQ ID NO:1), and

5'-CGATAGATCTTTATTTTGACTTATCTATTTCACAG-3' (SEQ ID NO:2).

Each of the above primers introduces a BglII site at the ends for cloning into the expression vector. Additionally, the sense strand primer introduces a KpnI site 14 nucleotides downstream from the signal peptide cleavage site, and the nonsense strand primer introduces a stop codon after Lys at position 48. The template DNA used was a clone of full length mature huPA in a yeast expression vector, as an alpha-factor fusion (pAB24UK300, consisting of the yeast shuttle vector pAB24 having a cassette inserted at the BamHI site, the cassette containing

the ADH2-GAP hybrid promoter, the yeast α-factor leader, the coding sequence for mature human uPA, and the GAP terminator, obtained from P. Valenzuela, Chiron Corporation) derived from a human kidney cDNA library (M.A. Truett *et al.*, DNA (1985) 4:333-49). Polymerase chain reactions were carried out in 100 μL volumes with the following components: 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dATP, dCTP, dGTP, and dTTP, 1 μM each primer, 9 ng template plasmid, and 2.5 U *Taq* DNA polymerase. The reaction conditions were 94°C for 1 min, followed by 37°C for 2 min, then 72°C for 3 min, for 30 cycles. Both the PCR fragment and a subcloning vector (pCBR, described by Frederik *et al.*, J Biol Chem (1990) 265:3793) containing the yeast expression cassette were digested with BglII and then ligated together, after treatment of the pCBR vector with alkaline phosphatase. Once the subclone was obtained (pCBRuPAα13), the expression cassette was isolated via BamHI digestion and ligated into the yeast shuttle vector (pAB24) to yield pAB24α13uPA1-48.

The expression plasmid was transformed into *Saccharomyces cerevisiae* AB110 (MATα leu2-3 -112 ura3 -52 pep4 -3 [cir]°) using the lithium acetate method (Ito *et al.*, J Bacteriol (1983) 153:163), and selected for uracil prototrophy. The plasmid copy number was then amplified by growth on minimal media without leucine, containing 8% glucose to keep ADH2-GAP promoter-mediated expression repressed. High level expression of secreted $huPA_{1-48}$ was obtained with pAB24α13uPA1-48 transformants of AB110 grown in leu^- medium and inoculating at 1:10 into YEP 4% glucose medium. All yeast cultures were grown at 30°C, 275 rpm, for 96 hours.

Example 2

(Purification of $huPA_{1-48}$)

One liter of yeast supernatant was harvested by centrifuging the cells at 2600 × g. Protein was concentrated from the supernatant by adding 70%

ammonium sulfate, incubating for 1 hr at 4°C, and separating the protein precipitate by centrifuging at 11,000 × g for 1 hr at 4°C. The protein pellets were resuspended in buffer containing 20 mM potassium phosphate, pH 7.0, 50 mM NaCl, and 1 mM EDTA. The suspension was dialyzed against the same buffer, with two changes of 4 L, overnight at 4°C. The entire dialysate was loaded onto a 1.8 L Sephadex® G-50 column at room temperature. Fractions were collected and monitored with UV at 254 nm, then pooled based on 16% Tris-Tricine SDS-PAGE (Novex) under non-reducing conditions. The peak fractions, containing monomeric $huPA_{1-48}$, were then loaded onto a 22 mm C18 reverse phase HPLC column (Vydac) and the protein eluted with a 0.6% gradient of acetonitrile containing 1% TFA. The major peak eluting at 33.5 minutes was collected and lyophilized. The purification yield is summarized in Table 1:

TABLE 1: Purification of $huPA_{1-48}$

Sample	Total Protein	Total Units[b] Yield
Crude supernatant	~200 mg[a]	3.3×10^6--
Ammonium sulfate	160 mg	2.0×10^6 60%
G50 Column	103 mg	1.3×10^6 42%
HPLC Purified	8.4 mg	7.4×10^5 22%

a) Estimated protein concentration due to interference with BCA assay

b) Unit = volume of crude sample required to inhibit binding of ^{125}I-ATF 50% in competition with biotinylated suPAR.

Example 3

(Characterization of $huPA_{1-48}$)

Purified $huPA_{1-48}$ was subjected to amino acid analysis and N-terminal sequencing, yielding the expected composition and sequence. The Edman

degradation was performed through residue 20. A stoichiometric amount of threonine was observed at cycle 18, indicating that this residue was not modified by fucosylation, as is found for uPA purified from eukaryotic cells. The absence of post translational modification was later confirmed by electrospray mass spectrometry. The binding activity of the recombinant $huPA_{1-48}$ was determined using a radio-receptor binding assay.

Baculovirus-derived recombinant human urokinase receptor was expressed as a truncated, soluble molecule as described previously for mouse L-cells (Masucci *et al.*, J Biol Chem (1991) 266:8655). The purified receptor was biotinylated with NHS-biotin, and immobilized at 1 μg/mL in PBS/0.1% BSA on streptavidin coated 96-well plates. Human uPA ATF (residues 1-135, obtained from M. Shuman, University of California, San Francisco) was iodinated using the Iodogen method (Pierce), and used as tracer. The ^{125}I-ATF was incubated at 100-500 pM with increasing amounts of $huPA_{1-48}$ in triplicate (100 pM - 1 μM) for 2 hours at room temperature in 0.1% BSA/PBS in a total volume of 200 μL. The plates were then washed 3 times with PBS/BSA, and the remaining bound radioactivity determined. The apparent K_d observed for $huPA_{1-48}$ was 0.3 nM, comparable to that reported for ATF and intact uPA.

Example 4

(Construction of $huPA_{1-48}$ Muteins)

In order to efficiently analyze the features of $huPA_{1-48}$, we performed a series of mutagenesis experiments utilizing phage display. Attempts to employ the system described by Scott and Smith, Science (1990) 249:386-90, were not successful. However, the use of monovalent phage display, using a phagemid and helper phage as described by Lowman *et al.*, Biochem (1991) 30:10832-38, did result in functional display of the protein domain. Finally, we employed an affinity epitope "tag" to reduce the fraction of phage bearing only wild-type pIII protein, reducing the background in panning experiments.

A.) Construction of Phagemids:

The starting materials were a phagemid construct (pGMEGF) comprising a human epidermal growth factor (hEGF) gene linked to the lac promoter, using pBluescript (Stratagene) as the backbone. The polylinker region of the vector contained within a PvuII fragment was replaced by a cassette comprising a leader sequence from the photobacterial superoxide dismutase fused to a synthetic gene for hEGF, in turn fused to residues 198-406 of the M13 pIII gene. The sequence of the insert is shown in SEQ ID NO:3. A synthetic gene encoding human urokinase residues 1-48 was obtained from J. Stratton-Thomas, Chiron Corporation.

Fusion proteins were generated using PCR. A first set of primers EUKMPCR1 and EUKGPCR1 were used with primer EUKPCR2 to add epitope tags to $huPA_{1-48}$ at the N-terminus, and to add an amber codon (TAG) and a BamHI site within residues 249-254 of the pIII protein at the C-terminus.

EUKMPCR1: CTCATCAAGCTTTAGCGGACTACAAAGACGATGACGATAAGA-GCAATGAACTTCATCAAG (SEQ ID NO:5);

EUKGPCR1: CTCATCAAGCTTTAGCCGAATACATGCCAATGGAAAGCAATGA-ACTTCATCAAG (SEQ ID NO:6);

EUKPCR2: CACCGGAACCGGATCCACCCTATTTTGACTTATC (SEQ ID NO:7).

The PCR reactions yielded primary products of the expected sizes, 204 and 197 bp.

A second set of primers, SRO1 and EUKCPCR1, were used with the EGF-containing phagemid construct as template. These primers added a BamHI site at pIII residues 250-251 and amplified a fragment ending at the unique ClaI site at residues 297-299 of pIII.

SRO1: GAAATAGATAAGTCAAAATAGGGTGGATCCGGTTCCGGTGATTTTGA-TTATG (SEQ ID NO:8); and

EUKCPCR1: GAAACCATCGATAGCAGCACCG (SEQ ID NO:9).

This PCR reaction yielded a primary product of approximately 180 bp. The PCR reaction products were separated from unreacted primers by size exclusion chromatography (Chromaspin-100, Clontech), digested with restriction enzymes Hd3 and BamHI (set 1) or BamHI and Cla1 (set 2), and isolated from a 2.5% agarose gel, using the Mermaid procedure (Bio-101). Each of the set 1 fragments were ligated with the C-terminal reaction 2 fragment, the ligations digested with Hd3 and Cla1, and the resulting fragments ligated into pGMEGF (digested with Hd3 and Cla1, dephosphorylated with alkaline phosphatase). The ligations were transformed into *E. coli* JS5 (Biorad) by electroporation. Strain JS5 overproduces lac repressor, and is sup0, preventing expression of the uPA_{1-48}-pIII fusion protein due to the amber stop codon between the uPA_{1-48} and pIII genes. Correct clones were identified by restriction analysis and confirmed by DNA sequencing. These steps yielded phagemids pHM1a (M1Flag-uPA_{1-48}) and pHM3a (Glutag-uPA_{1-48}). The DNA sequences of the fusion proteins in these phagemids are shown in SEQ ID NO:10 and SEQ ID NO:12.

The phagemid containing a synthetic gene for uPA_{1-48} was constructed in the same vector by the following steps. The sequence of the synthetic gene is shown in SEQ ID NO:14. Plasmid pCBRuPA (16 μg), a derivative of pCBR (Frederick *et al.*, J Biol Chem (1990) 265:3793) containing this synthetic gene for uPA_{1-48}, inserted between the yeast α-factor leader and GAPDH terminator as a BglII fragment, was digested with Sac1 and Cla1, and adapted for phagemid expression using the following set of synthetic oligonucleotides:

SRO35: AGCTTTAGCGGAATACATGCCAATGGAAAGCAATGAGCT (SEQ ID NO:16);

SRO36: CATTGCTTTCCATTGGCATGTATTCCGCTAA (SEQ ID NO:17);

SRO37: CGATAAGTCAAAATAGGGTG (SEQ ID NO:18); and

SRO38: GATCCACCCTATTTTGACTTAT (SEQ ID NO:19).

Oligonucleotides SRO36 and SRO37 (250 pmol) were phosphorylated with polynucleotide kinase and annealed with equimolar amounts of oligos SRO35 and

SRO38, respectively. The two annealed duplexes (125 pmol) were ligated overnight with the digested plasmid DNA, the ligase heat inactivated, and the ends phosphorylated with polynucleotide kinase. The DNA was run on a 6% polyacrylamide gel and the correct sized band (ca. 200 bp) was excised and isolated. The insert was ligated with plasmid pHM1a (digested with Hd3 and BamHI) and phosphatased, and the ligations transformed into *E. coli* JS5. The correct recombinants were identified by restriction analysis, and confirmed by DNA sequencing, yielding phagemid pHM3-3.

B.) Production and Panning of Phagemids:

To produce phagemid particles, DNAs were transformed into *E. coli* strain XL1-blue (Stratagene) by electroporation. This strain was used because it is supE44 (TAG codon encodes Gln), laciQ (overproduces lac repressor), and makes phage (F'+). Overnight cultures were grown in 2× YT broth containing 50 μg/mL ampicillin and 10 μg/mL tetracycline (to maintain the F'). Cells were diluted 1:50 or 1:100 into the same media, grown for 20 minutes as 37°C for 10 minutes at 225 rpm to enhance phage attachment, and then grown with normal agitation at 325 rpm overnight. Phage particles were then purified and concentrated by two successive precipitations with polyethylene glycol. The concentrations of phage present were determined by infection of *E. coli* XL1-blue and plating on L broth plates containing 50 μg/mL ampicillin.

To pan for binding phage particles, small tissue culture plates were coated either with anti-Glu antibody (R. Clark, Onyx Corporation) or streptavidin at 10 μg/mL in PBS overnight. Plates were then blocked with PBS containing 0.1% BSA. To the streptavidin plates was then added 1 μg/mL of biotinylated secreted human urokinase receptor obtained by recombinant baculovirus infection of *A. californica* Sf9 cells. After 2 hours at room temperature, the plates were again blocked with BSA, and phage (10^6-10^{10} cfu) were added in 1 mL of PBS/BSA. After incubation for 1 hour, non-specifically adhered phage were removed by washing (7× 1 mL PBS/BSA), and the remaining phage eluted with

1 mL of 0.1 M glycine, pH 2.2, for 30 minutes. The eluted phage were immediately neutralized with 1 M Tris, pH 9.4, and stored at 4°C overnight. The number of phage eluted was determined by titering on *E. coli* XL1-blue on ampicillin plates. The procedure, where phage are first bound and eluted from the Glu-Ab plates and then panned against receptor plates, reduces the high background that would otherwise result from the large number of phage containing only wild type pIII: only phage containing an insert in pIII display an epitope tag and are selected on anti-Glu MAbs plates.

Table 2 shows that phagemids displaying uPA_{1-48} are specifically bound and eluted from immobilized urokinase receptor. Table 3 demonstrates that the phagemid which displays a Glu tag-uPA_{1-48} fusion is specifically retained by immobilized Glu Ab. Finally, Table 4 shows that a population of the Glu-uPA_{1-48} phagemid which has been specifically eluted from the Glu Ab plates, is retained with a much higher yield on urokinase receptor plates, than is the unenriched phagemid population.

TABLE 2: Panning on Immobilized Receptor

Sample	Phage/phagemid	Input[e]	%Yield -uPAR	%Yield +uPAR
1[a]	1a	9.4×10^9	0.0018	0.094
2[b]	3a	1.4×10^{10}	0.0014	0.08
3[c]	pGMEGF	1.3×10^{10}	0.0015	0.0012
4[d]	LP67 (control)	1.4×10^9	-	0.0099

[a] M1-FLAG-UPAELD-short pIII (pHM1a)
[b] Glu-tag-UPAELD-short pIII (pHM3a)
[c] M1-FLAG-EGF-medium long pIII (pGMEGF)
[d] LP67 - control phage (Amp^r M13)
[e] ampicillin resistant colonies, in cfu

TABLE 3: Panning phage with Glu-Ab or suPAR

Sample	Phage/phagemid	Input[a]	%Yield suPAR[b]	%Yield GluAb
1	pHM1a	1.5×10^{10}	0.55%	0.003%
2	pHM3a	2.5×10^{10}	0.44%	0.048%
3	LP67 (control)	3.5×10^{5}	0.008%	-

[a] ampicillin resistant colonies, in cfu

[b] soluble uPA receptor

TABLE 4: Panning GluAb-unenriched and enriched phage on suPAR

Sample	Phage/phagemid	Input[a]	%Yield suPAR[b]	%Yield GluAb
1	pHM3a	2.7×10^{7}	0.85%	0.08%
2	pHM3a (enriched)	6×10^{6}	9.7%	3.3%
3	LP67 (control)	5.4×10^{6}	<0.04%	<0.02%

[a] ampicillin resistant colonies, in cfu

[b] soluble uPA receptor

These enriched phagemid pools are used for multiple mutagenesis strategies in order to identify improved uPA_{1-48} ligands with altered specificity or improved affinity. For example the region between residues 13 and 32 of human uPA has been implicated in receptor binding (E. Appella *et al.*, J Biol Chem (1987) 262:4437-40). Key residues in the region from 19-30 can be easily mutated by replacing the region between the unique restriction sites KpnI and MunI.

In order to rapidly and quantitatively assess the binding affinities of the resulting uPA_{1-48} variants, relatively large quantities of properly folded proteins are required. Although this could be done by bacterial expression, using the phagemid constructs in a sup0 strain and inducing with IPTG, such a strategy yields relatively small amounts of protein in the periplasm. A second strategy is to express the variants in yeast, as described above for the wild type protein. To accomplish this we have constructed a yeast expression vector which enables us to move fragments encoding residues 4-48 of uPA_{1-48} in a single step from the phagemid vectors. This was accomplished as follows: Plasmid pAGαG,

identical to pCBR except for a small deletion of an Xba fragment in the ADH2-GAPDH promoter, was digested with Sac1, which cleaves once within the promoter, and then treated with Mung Bean nuclease which destroys the site. Subsequent religation yielded plasmid pAGαG-Sac. Digestion with BglII and treatment with alkaline phosphatase yielded a vector into which was ligated the BglII fragment corresponding to the synthetic gene for uPA_{1-48}. Transformation of *E. coli* strain HB101 to ampicillin resistance and restriction analysis yielded the correct clone. The 2.4 kB BamHI fragment from this plasmid (pAGαG-SacI-48synth), containing the expression cassette, was isolated and ligated into pAB24, which had been treated with BamHI and alkaline phosphatase. The resulting plasmid has unique SacI and XhoI sites which can be used for transfer of the phagemid 1-48 genes. This is accomplished by digesting the phagemid with BamHI, treating with Mung Bean Nuclease, digesting with SacI and isolating the 145 bp fragment. The vector is digested with XhoI, treated with Mung Bean Nuclease, digested with SacI,and treated with alkaline phosphatase. Ligation then yields the correct recombinants in a single step in the yeast expression vector. Transformation of yeast strain AB110 then yields high levels of secreted 1-48 variants for analysis.

Using this construct, one can express a library of uPA variations for screening. Variations may be constructed by a variety of methods, including low-fidelity PCR (which introduces a large number of random point mutations), site-specific mutation, primer-based mutagenesis, and ligation of the uPA_{1-48} sequence (or portions thereof) to a random oligonucleotide sequence (*e.g.*, by attaching $(NNS)_x$ to the uPA_{1-48} coding sequence, or substituting NNS for one or more uPA_{1-48} codons). Generation of random oligonucleotide sequences is detailed in Devlin, WO91/18980, incorporated herein by reference. Phage displaying uPA_{1-48} variants (having one or more amino acid substitutions) are screened according to the protocol described above (using, *e.g.*, pHM3a as a positive control) and selected for improved binding.

Example 5

(Formulation of $huPA_{1-48}$)

$huPA_{1-48}$ formulations suitable for use in chemotherapy are prepared as follows:

A) Injectable Formulation:

$huPA_{1-48}$	7.0 mg
Na_2HPO_4 (0.5 M)	0.5 mL
mannitol (25%)	2.5 mL
sodium laureate (1%)	2.5 mL
pH	7.5
PBS qs	20 mL

This formulation is prepared following the procedure set forth in US 4,816,440, incorporated herein by reference. The formulation is administered by parenteral injection at the site to be treated. The formulation is also generally suitable for administration as eyedrops directly to the conjunctiva, or by intranasal administration as an aerosol. Alternatively, a concentrated formulation (*e.g.*, reducing the phosphate buffered saline to 2 mL) may be used to fill an Alzet® minipump, and the minipump implanted at the site to be treated.

B) Ophthalmic Preparation:

$huPA_{1-48}$		1 mg
fibronectin		69 mg
albumin		37.5 mg
water	qs	3.0 mL
HCl (0.01 M)	qs	pH 4.0

This dosage form is prepared following the procedure set forth in US 5,124,155, incorporated herein by reference. The fibronectin and albumin are dissolved in water to form a 3.0 mL solution, and HCl added to a pH of 4.0, causing the fibronectin to flocculate. The flocculent is filtered, and combined with the $huPA_{1-48}$. The mixture is then placed in a contact lens mold, and the mold closed for 30 min to form a corneal "shield" in the shape of a contact lens.

The shield releases $huPA_{1-48}$ over a period of time, and is useful for preventing angiogenesis of corneal tissue following ophthalmic surgery.

The present invention has been described with reference to specific embodiments. However, this application is intended to cover those changes and substitutions which may be made by those skilled in the art without departing from the spirit and the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Rosenberg, Steven
Stratton-Thomas, Jennifer R.

(ii) TITLE OF INVENTION: Expression of Urokinase Plasminogen Activator Inhibitors

(iii) NUMBER OF SEQUENCES: 19

(iv) CORRESPONDENCE ADDRESS:
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(E) COUNTRY: USA
(F) ZIP: 94608

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Green, Grant D.
(B) REGISTRATION NUMBER: 31,259
(C) REFERENCE/DOCKET NUMBER: 0939.001

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 510-601-2706
(B) TELEFAX: 510-655-3542

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATGCTAGATC TAATGAACTT CATCAGGTAC CATCG
35

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CGATAGATCT TTATTTTGAC TTATCTATTT CACAG

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 953 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: M1Flag-EGF-pIII fusion

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 25..903

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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                             Met Asn Lys Ala Lys Thr Leu Leu Phe
                               1               5

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Thr Ala Leu Ala Phe Gly Leu Ser His Gln Ala Leu Ala Asp Tyr Lys
 10                  15                  20                  25

GAC GAT GAC GAT AAG AAT TCT GAC AGT GAA TGC CCG CTG AGC CAC GAC
Asp Asp Asp Asp Lys Asn Ser Asp Ser Glu Cys Pro Leu Ser His Asp
                    30                  35                  40

GGC TAC TGC CTG CAC GAC GGT GTT TGC ATG TAC ATC GAA GCT CTA GAC
Gly Tyr Cys Leu His Asp Gly Val Cys Met Tyr Ile Glu Ala Leu Asp
                45                  50                  55
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AAG TAC GCA TGC AAC TGC GTT GTT GGG TAC ATC GGT GAG CGC TGC CAG
243
Lys Tyr Ala Cys Asn Cys Val Val Gly Tyr Ile Gly Glu Arg Cys Gln
          60                  65                  70

TAC CGA GAT CTT AAG TGG TGG GAA CTC CGT GGG CCC TTC GTT TGT GAA
291
Tyr Arg Asp Leu Lys Trp Trp Glu Leu Arg Gly Pro Phe Val Cys Glu
     75                  80                  85

TAT CAA GGC CAA TCG TCT GAC CTG CCT CAA CCT CCT GTC AAT GCT GGC
339
Tyr Gln Gly Gln Ser Ser Asp Leu Pro Gln Pro Pro Val Asn Ala Gly
 90                  95                 100                 105

GGC GGC TCT GGT GGT GGT TCT GGT GGC GGC TCT GAG GGT GGT GGC TCT
387
Gly Gly Ser Gly Gly Gly Ser Gly Gly Gly Ser Glu Gly Gly Gly Ser
                110                 115                 120

GAG GGT GGC GGT TCT GAG GGT GGC GGC TCT GAG GGA GGC GGT TCC GGT
435
Glu Gly Gly Gly Ser Glu Gly Gly Gly Ser Glu Gly Gly Gly Ser Gly
            125                 130                 135

GGT GGC TCT GGT TCC GGT GAT TTT GAT TAT GAA AAG ATG GCA AAC GCT
483
Gly Gly Ser Gly Ser Gly Asp Phe Asp Tyr Glu Lys Met Ala Asn Ala
        140                 145                 150

AAT AAG GGG GCT ATG ACC GAA AAT GCC GAT GAA AAC GCG CTA CAG TCT
531
Asn Lys Gly Ala Met Thr Glu Asn Ala Asp Glu Asn Ala Leu Gln Ser
    155                 160                 165

GAC GCT AAA GGC AAA CTT GAT TCT GTC GCT ACT GAT TAC GGT GCT GCT
579
Asp Ala Lys Gly Lys Leu Asp Ser Val Ala Thr Asp Tyr Gly Ala Ala
170                 175                 180                 185

ATC GAT GGT TTC ATT GGT GAC GTT TCC GGC CTT GCT AAT GGT AAT GGT
627
Ile Asp Gly Phe Ile Gly Asp Val Ser Gly Leu Ala Asn Gly Asn Gly
                190                 195                 200

GCT ACT GGT GAT TTT GCT GGC TCT AAT TCC CAA ATG GCT CAA GTC GGT
675
Ala Thr Gly Asp Phe Ala Gly Ser Asn Ser Gln Met Ala Gln Val Gly
            205                 210                 215

GAC GGT GAT AAT TCA CCT TTA ATG AAT AAT TTC CGT CAA TAT TTA CCT
723
Asp Gly Asp Asn Ser Pro Leu Met Asn Asn Phe Arg Gln Tyr Leu Pro
        220                 225                 230

TCC CTC CCT CAA TCG GTT GAA TGT CGC CCT TTT GTC TTT AGC GCT GGT
771
Ser Leu Pro Gln Ser Val Glu Cys Arg Pro Phe Val Phe Ser Ala Gly
    235                 240                 245
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AAA CCA TAT GAA TTT TCT ATT GAT TGT GAC AAA ATA AAC TTA TTC CGT
819
Lys Pro Tyr Glu Phe Ser Ile Asp Cys Asp Lys Ile Asn Leu Phe Arg
250                 255                 260                 265

GGT GTC TTT GCG TTT CTT TTA TAT GTT GCC ACC TTT ATG TAT GTA TTT
867
Gly Val Phe Ala Phe Leu Leu Tyr Val Ala Thr Phe Met Tyr Val Phe
                270                 275                 280

TCT ACG TTT GCT AAC ATA CTG CGT AAT AAG GAG TCT TAATCATGCG
913
Ser Thr Phe Ala Asn Ile Leu Arg Asn Lys Glu Ser
            285                 290

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953
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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 293 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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            20                  25                  30

Asp Ser Glu Cys Pro Leu Ser His Asp Gly Tyr Cys Leu His Asp Gly
        35                  40                  45

Val Cys Met Tyr Ile Glu Ala Leu Asp Lys Tyr Ala Cys Asn Cys Val
    50                  55                  60

Val Gly Tyr Ile Gly Glu Arg Cys Gln Tyr Arg Asp Leu Lys Trp Trp
65                  70                  75                  80

Glu Leu Arg Gly Pro Phe Val Cys Glu Tyr Gln Gly Gln Ser Ser Asp
                85                  90                  95

Leu Pro Gln Pro Pro Val Asn Ala Gly Gly Gly Ser Gly Gly Gly Ser
            100                 105                 110

Gly Gly Gly Ser Glu Gly Gly Gly Ser Glu Gly Gly Gly Ser Glu Gly
        115                 120                 125

Gly Gly Ser Glu Gly Gly Gly Ser Gly Gly Gly Ser Gly Ser Gly Asp
    130                 135                 140

Phe Asp Tyr Glu Lys Met Ala Asn Ala Asn Lys Gly Ala Met Thr Glu
145                 150                 155                 160

Asn Ala Asp Glu Asn Ala Leu Gln Ser Asp Ala Lys Gly Lys Leu Asp
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165 170 175

Ser Val Ala Thr Asp Tyr Gly Ala Ala Ile Asp Gly Phe Ile Gly Asp
180 185 190

Val Ser Gly Leu Ala Asn Gly Asn Gly Ala Thr Gly Asp Phe Ala Gly
195 200 205

Ser Asn Ser Gln Met Ala Gln Val Gly Asp Gly Asp Asn Ser Pro Leu
210 215 220

Met Asn Asn Phe Arg Gln Tyr Leu Pro Ser Leu Pro Gln Ser Val Glu
225 230 235 240

Cys Arg Pro Phe Val Phe Ser Ala Gly Lys Pro Tyr Glu Phe Ser Ile
245 250 255

Asp Cys Asp Lys Ile Asn Leu Phe Arg Gly Val Phe Ala Phe Leu Leu
260 265 270

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(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: EUKMPCR1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CTCATCAAGC TTTAGCGGAC TACAAAGACG ATGACGATAA GAGCAATGAA CTTCATCAAG
60

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 54 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: EUKGPCR1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CTCATCAAGC TTTAGCCGAA TACATGCCAA TGGAAAGCAA TGAACTTCAT CAAG
54

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: EUKPCR2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CACCGGAACC GGATCCACCC TATTTTGACT TATC
34

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 52 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: SRO1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GAAATAGATA AGTCAAAATA GGGTGGATCC GGTTCCGGTG ATTTTGATTA TG
52

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: EUKCPCR1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GAAACCATCG ATAGCAGCAC CG
22

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 779 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: M1Flag uPA1-48 - pIII fusion

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 25..729

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

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1 5

ACT GCG CTA GCT TTT GGT TTA TCT CAT CAA GCT TTA GCC GAC TAC AAA
99
Thr Ala Leu Ala Phe Gly Leu Ser His Gln Ala Leu Ala Asp Tyr Lys
10 15 20 25

GAC GAT GAC GAT AAG AGC AAT GAA CTT CAT CAA GTT CCA TCG AAC TGT
147
Asp Asp Asp Asp Lys Ser Asn Glu Leu His Gln Val Pro Ser Asn Cys
30 35 40

GAC TGT CTA AAT GGA GGA ACA TGT GTG TCC AAC AAG TAC TTC TCC AAC
195
Asp Cys Leu Asn Gly Gly Thr Cys Val Ser Asn Lys Tyr Phe Ser Asn
45 50 55

ATT CAC TGG TGC AAC TGC CCA AAG AAA TTC GGA GGG CAG CAC TGT GAA
243
Ile His Trp Cys Asn Cys Pro Lys Lys Phe Gly Gly Gln His Cys Glu
60 65 70

```
ATA GAT AAG TCA AAA TAG GGT GGA TCC GGT TCC GGT GAT TTT GAT TAT
291
Ile Asp Lys Ser Lys  *  Gly Gly Ser Gly Ser Gly Asp Phe Asp Tyr
     75                 80                  85

GAA AAG ATG GCA AAC GCT AAT AAG GGG GCT ATG ACC GAA AAT GCC GAT
339
Glu Lys Met Ala Asn Ala Asn Lys Gly Ala Met Thr Glu Asn Ala Asp
 90                 95                 100                 105

GAA AAC GCG CTA CAG TCT GAC GCT AAA GGC AAA CTT GAT TCT GTC GCT
387
Glu Asn Ala Leu Gln Ser Asp Ala Lys Gly Lys Leu Asp Ser Val Ala
                110                 115                 120

ACT GAT TAC GGT GCT GCT ATC GAT GGT TTC ATT GGT GAC GTT TCC GGC
435
Thr Asp Tyr Gly Ala Ala Ile Asp Gly Phe Ile Gly Asp Val Ser Gly
            125                 130                 135

CTT GCT AAT GGT AAT GGT GCT ACT GGT GAT TTT GCT GGC TCT AAT TCC
483
Leu Ala Asn Gly Asn Gly Ala Thr Gly Asp Phe Ala Gly Ser Asn Ser
        140                 145                 150

CAA ATG GCT CAA GTC GGT GAC GGT GAT AAT TCA CCT TTA ATG AAT AAT
531
Gln Met Ala Gln Val Gly Asp Gly Asp Asn Ser Pro Leu Met Asn Asn
    155                 160                 165

TTC CGT CAA TAT TTA CCT TCC CTC CCT CAA TCG GTT GAA TGT CGC CCT
579
Phe Arg Gln Tyr Leu Pro Ser Leu Pro Gln Ser Val Glu Cys Arg Pro
170                 175                 180                 185

TTT GTC TTT AGC GCT GGT AAA CCA TAT GAA TTT TCT ATT GAT TGT GAC
627
Phe Val Phe Ser Ala Gly Lys Pro Tyr Glu Phe Ser Ile Asp Cys Asp
                190                 195                 200

AAA ATA AAC TTA TTC CGT GGT GTC TTT GCG TTT CTT TTA TAT GTT GCC
675
Lys Ile Asn Leu Phe Arg Gly Val Phe Ala Phe Leu Leu Tyr Val Ala
            205                 210                 215

ACC TTT ATG TAT GTA TTT TCT ACG TTT GCT AAC ATA CTG CGT AAT AAG
723
Thr Phe Met Tyr Val Phe Ser Thr Phe Ala Asn Ile Leu Arg Asn Lys
        220                 225                 230

GAG TCT TAATCATGCG CGCTCACTGG CCGTCGTTTT ACAACGTCGT GACTGGGAAA
779
Glu Ser
    235
```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 235 amino acids
(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
Met Asn Lys Ala Lys Thr Leu Leu Phe Thr Ala Leu Ala Phe Gly Leu
 1               5                  10                  15

Ser His Gln Ala Leu Ala Asp Tyr Lys Asp Asp Asp Asp Lys Ser Asn
            20                  25                  30

Glu Leu His Gln Val Pro Ser Asn Cys Asp Cys Leu Asn Gly Gly Thr
        35                  40                  45

Cys Val Ser Asn Lys Tyr Phe Ser Asn Ile His Trp Cys Asn Cys Pro
    50                  55                  60

Lys Lys Phe Gly Gly Gln His Cys Glu Ile Asp Lys Ser Lys  *  Gly
65                  70                  75                  80

Gly Ser Gly Ser Gly Asp Phe Asp Tyr Glu Lys Met Ala Asn Ala Asn
                85                  90                  95

Lys Gly Ala Met Thr Glu Asn Ala Asp Glu Asn Ala Leu Gln Ser Asp
            100                 105                 110

Ala Lys Gly Lys Leu Asp Ser Val Ala Thr Asp Tyr Gly Ala Ala Ile
        115                 120                 125

Asp Gly Phe Ile Gly Asp Val Ser Gly Leu Ala Asn Gly Asn Gly Ala
    130                 135                 140

Thr Gly Asp Phe Ala Gly Ser Asn Ser Gln Met Ala Gln Val Gly Asp
145                 150                 155                 160

Gly Asp Asn Ser Pro Leu Met Asn Asn Phe Arg Gln Tyr Leu Pro Ser
                165                 170                 175

Leu Pro Gln Ser Val Glu Cys Arg Pro Phe Val Phe Ser Ala Gly Lys
            180                 185                 190

Pro Tyr Glu Phe Ser Ile Asp Cys Asp Lys Ile Asn Leu Phe Arg Gly
        195                 200                 205

Val Phe Ala Phe Leu Leu Tyr Val Ala Thr Phe Met Tyr Val Phe Ser
    210                 215                 220

Thr Phe Ala Asn Ile Leu Arg Asn Lys Glu Ser
225                 230                 235
```

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 773 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: Glu-tag uPA1-48 - pIII fusion

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 25..723

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

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CCATGGCTAC AGAGGAATAT TAAA ATG AAT AAG GCA AAA ACT TTA CTC TTC
 51
                            Met Asn Lys Ala Lys Thr Leu Leu Phe
                              1               5

ACT GCG CTA GCT TTT GGT TTA TCT CAT CAA GCT TTA GCC GAA TAC ATG
 99
Thr Ala Leu Ala Phe Gly Leu Ser His Gln Ala Leu Ala Glu Tyr Met
 10                  15                  20                  25

CCA ATG GAA AGC AAT GAA CTT CAT CAA GTT CCA TCG AAC TGT GAC TGT
147
Pro Met Glu Ser Asn Glu Leu His Gln Val Pro Ser Asn Cys Asp Cys
                30                  35                  40

CTA AAT GGA GGA ACA TGT GTG TCC AAC AAG TAC TTC TCC AAC ATT CAC
195
Leu Asn Gly Gly Thr Cys Val Ser Asn Lys Tyr Phe Ser Asn Ile His
            45                  50                  55

TGG TGC AAC TGC CCA AAG AAA TTC GGA GGG CAG CAC TGT GAA ATA GAT
243
Trp Cys Asn Cys Pro Lys Lys Phe Gly Gly Gln His Cys Glu Ile Asp
        60                  65                  70

AAG TCA AAA TAG GGT GGA TCC GGT TCC GGT GAT TTT GAT TAT GAA AAG
291
Lys Ser Lys  *  Gly Gly Ser Gly Ser Gly Asp Phe Asp Tyr Glu Lys
    75              80                  85

ATG GCA AAC GCT AAT AAG GGG GCT ATG ACC GAA AAT GCC GAT GAA AAC
339
Met Ala Asn Ala Asn Lys Gly Ala Met Thr Glu Asn Ala Asp Glu Asn
 90                  95                  100                 105

GCG CTA CAG TCT GAC GCT AAA GGC AAA CTT GAT TCT GTC GCT ACT GAT
387
Ala Leu Gln Ser Asp Ala Lys Gly Lys Leu Asp Ser Val Ala Thr Asp
                110                 115                 120

TAC GGT GCT GCT ATC GAT GGT TTC ATT GGT GAC GTT TCC GGC CTT GCT
435
Tyr Gly Ala Ala Ile Asp Gly Phe Ile Gly Asp Val Ser Gly Leu Ala
            125                 130                 135

AAT GGT AAT GGT GCT ACT GGT GAT TTT GCT GGC TCT AAT TCC CAA ATG
483
Asn Gly Asn Gly Ala Thr Gly Asp Phe Ala Gly Ser Asn Ser Gln Met
```

```
                140                 145                 150

     GCT CAA GTC GGT GAC GGT GAT AAT TCA CCT TTA ATG AAT AAT TTC CGT
     531
     Ala Gln Val Gly Asp Gly Asp Asn Ser Pro Leu Met Asn Asn Phe Arg
     155                 160                 165

     CAA TAT TTA CCT TCC CTC CCT CAA TCG GTT GAA TGT CGC CCT TTT GTC
     579
     Gln Tyr Leu Pro Ser Leu Pro Gln Ser Val Glu Cys Arg Pro Phe Val
     170                 175                 180                 185

     TTT AGC GCT GGT AAA CCA TAT GAA TTT TCT ATT GAT TGT GAC AAA ATA
     627
     Phe Ser Ala Gly Lys Pro Tyr Glu Phe Ser Ile Asp Cys Asp Lys Ile
                     190                 195                 200

     AAC TTA TTC CGT GGT GTC TTT GCG TTT CTT TTA TAT GTT GCC ACC TTT
     675
     Asn Leu Phe Arg Gly Val Phe Ala Phe Leu Leu Tyr Val Ala Thr Phe
                 205                 210                 215

     ATG TAT GTA TTT TCT ACG TTT GCT AAC ATA CTG CGT AAT AAG GAG TCT
     723
     Met Tyr Val Phe Ser Thr Phe Ala Asn Ile Leu Arg Asn Lys Glu Ser
             220                 225                 230

     TAATCATGCG CGCTCACTGG CCGTCGTTTT ACAACGTCGT GACTGGGAAA
     773
```

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 233 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```
Met Asn Lys Ala Lys Thr Leu Leu Phe Thr Ala Leu Ala Phe Gly Leu
  1               5                  10                  15

Ser His Gln Ala Leu Ala Glu Tyr Met Pro Met Glu Ser Asn Glu Leu
            20                  25                  30

His Gln Val Pro Ser Asn Cys Asp Cys Leu Asn Gly Gly Thr Cys Val
        35                  40                  45

Ser Asn Lys Tyr Phe Ser Asn Ile His Trp Cys Asn Cys Pro Lys Lys
    50                  55                  60

Phe Gly Gly Gln His Cys Glu Ile Asp Lys Ser Lys  *  Gly Gly Ser
65                  70                  75                  80

Gly Ser Gly Asp Phe Asp Tyr Glu Lys Met Ala Asn Ala Asn Lys Gly
                85                  90                  95

Ala Met Thr Glu Asn Ala Asp Glu Asn Ala Leu Gln Ser Asp Ala Lys
```

100 105 110

Gly Lys Leu Asp Ser Val Ala Thr Asp Tyr Gly Ala Ala Ile Asp Gly
115 120 125

Phe Ile Gly Asp Val Ser Gly Leu Ala Asn Gly Asn Gly Ala Thr Gly
130 135 140

Asp Phe Ala Gly Ser Asn Ser Gln Met Ala Gln Val Gly Asp Gly Asp
145 150 155 160

Asn Ser Pro Leu Met Asn Asn Phe Arg Gln Tyr Leu Pro Ser Leu Pro
165 170 175

Gln Ser Val Glu Cys Arg Pro Phe Val Phe Ser Ala Gly Lys Pro Tyr
180 185 190

Glu Phe Ser Ile Asp Cys Asp Lys Ile Asn Leu Phe Arg Gly Val Phe
195 200 205

Ala Phe Leu Leu Tyr Val Ala Thr Phe Met Tyr Val Phe Ser Thr Phe
210 215 220

Ala Asn Ile Leu Arg Asn Lys Glu Ser
225 230

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 773 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: Glu-tag uPA1-48 synth. - pIII map

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 25..723

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CCATGGCTAC AGAGGAATAT TAAA ATG AAT AAG GCA AAA ACT TTA CTC TTC
51
Met Asn Lys Ala Lys Thr Leu Leu Phe
1 5

ACT GCG CTA GCT TTT GGT TTA TCT CAT CAA GCT TTA GCG GAA TAC ATG
99
Thr Ala Leu Ala Phe Gly Leu Ser His Gln Ala Leu Ala Glu Tyr Met
10 15 20 25

CCA ATG GAA AGC AAT GAG CTC CAT CAA GTA CCA TCG AAC TGT GAC TGT
147

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      Pro Met Glu Ser Asn Glu Leu His Gln Val Pro Ser Asn Cys Asp Cys
                          30                  35                  40

      CTA AAT GGA GGT ACC TGT GTG TCC AAC AAG TAC TTT TCG AAC ATT CAC
 5    195
      Leu Asn Gly Gly Thr Cys Val Ser Asn Lys Tyr Phe Ser Asn Ile His
                    45                  50                  55

      TGG TGC AAT TGC CCA AAG AAA TTC GGA GGG CAG CAC TGT GAA ATC GAT
10    243
      Trp Cys Asn Cys Pro Lys Lys Phe Gly Gly Gln His Cys Glu Ile Asp
              60                  65                  70

      AAG TCA AAA TAG GGT GGA TCC GGT TCC GGT GAT TTT GAT TAT GAA AAG
15    291
      Lys Ser Lys  *  Gly Gly Ser Gly Ser Gly Asp Phe Asp Tyr Glu Lys
           75                  80                  85

      ATG GCA AAC GCT AAT AAG GGG GCT ATG ACC GAA AAT GCC GAT GAA AAC
20    339
      Met Ala Asn Ala Asn Lys Gly Ala Met Thr Glu Asn Ala Asp Glu Asn
       90                  95                  100                 105

      GCG CTA CAG TCT GAC GCT AAA GGC AAA CTT GAT TCT GTC GCT ACT GAT
25    387
      Ala Leu Gln Ser Asp Ala Lys Gly Lys Leu Asp Ser Val Ala Thr Asp
                    110                 115                 120

      TAC GGT GCT GCT ATC GAT GGT TTC ATT GGT GAC GTT TCC GGC CTT GCT
30    435
      Tyr Gly Ala Ala Ile Asp Gly Phe Ile Gly Asp Val Ser Gly Leu Ala
              125                 130                 135

      AAT GGT AAT GGT GCT ACT GGT GAT TTT GCT GGC TCT AAT TCC CAA ATG
35    483
      Asn Gly Asn Gly Ala Thr Gly Asp Phe Ala Gly Ser Asn Ser Gln Met
           140                 145                 150

      GCT CAA GTC GGT GAC GGT GAT AAT TCA CCT TTA ATG AAT AAT TTC CGT
40    531
      Ala Gln Val Gly Asp Gly Asp Asn Ser Pro Leu Met Asn Asn Phe Arg
       155                 160                 165

      CAA TAT TTA CCT TCC CTC CCT CAA TCG GTT GAA TGT CGC CCT TTT GTC
45    579
      Gln Tyr Leu Pro Ser Leu Pro Gln Ser Val Glu Cys Arg Pro Phe Val
      170                 175                 180                 185

      TTT AGC GCT GGT AAA CCA TAT GAA TTT TCT ATT GAT TGT GAC AAA ATA
50    627
      Phe Ser Ala Gly Lys Pro Tyr Glu Phe Ser Ile Asp Cys Asp Lys Ile
                    190                 195                 200

      AAC TTA TTC CGT GGT GTC TTT GCG TTT CTT TTA TAT GTT GCC ACC TTT
55    675
      Asn Leu Phe Arg Gly Val Phe Ala Phe Leu Leu Tyr Val Ala Thr Phe
                    205                 210                 215

      ATG TAT GTA TTT TCT ACG TTT GCT AAC ATA CTG CGT AAT AAG GAG TCT
60    723
      Met Tyr Val Phe Ser Thr Phe Ala Asn Ile Leu Arg Asn Lys Glu Ser
```

220 225 230

TAATCATGCG CGCTCACTGG CCGTCGTTTT ACAACGTCGT GACTGGGAAA
773

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 233 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Met Asn Lys Ala Lys Thr Leu Leu Phe Thr Ala Leu Ala Phe Gly Leu
1 5 10 15

Ser His Gln Ala Leu Ala Glu Tyr Met Pro Met Glu Ser Asn Glu Leu
20 25 30

His Gln Val Pro Ser Asn Cys Asp Cys Leu Asn Gly Gly Thr Cys Val
35 40 45

Ser Asn Lys Tyr Phe Ser Asn Ile His Trp Cys Asn Cys Pro Lys Lys
50 55 60

Phe Gly Gly Gln His Cys Glu Ile Asp Lys Ser Lys * Gly Gly Ser
65 70 75 80

Gly Ser Gly Asp Phe Asp Tyr Glu Lys Met Ala Asn Ala Asn Lys Gly
85 90 95

Ala Met Thr Glu Asn Ala Asp Glu Asn Ala Leu Gln Ser Asp Ala Lys
100 105 110

Gly Lys Leu Asp Ser Val Ala Thr Asp Tyr Gly Ala Ala Ile Asp Gly
115 120 125

Phe Ile Gly Asp Val Ser Gly Leu Ala Asn Gly Asn Gly Ala Thr Gly
130 135 140

Asp Phe Ala Gly Ser Asn Ser Gln Met Ala Gln Val Gly Asp Gly Asp
145 150 155 160

Asn Ser Pro Leu Met Asn Asn Phe Arg Gln Tyr Leu Pro Ser Leu Pro
165 170 175

Gln Ser Val Glu Cys Arg Pro Phe Val Phe Ser Ala Gly Lys Pro Tyr
180 185 190

Glu Phe Ser Ile Asp Cys Asp Lys Ile Asn Leu Phe Arg Gly Val Phe
195 200 205

Ala Phe Leu Leu Tyr Val Ala Thr Phe Met Tyr Val Phe Ser Thr Phe
210 215 220

Ala Asn Ile Leu Arg Asn Lys Glu Ser
225 230

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: SRO35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

AGCTTTAGCG GAATACATGC CAATGGAAAG CAATGAGCT
39

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: SRO36

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CATTGCTTTC CATTGGCATG TATTCCGCTA A
31

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: SRO37

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CGATAAGTCA AAATAGGGTG
20

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: SRO38

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GATCCACCCT ATTTTGACTT AT
22

WHAT IS CLAIMED:

1. A method for producing a non-fucosylated polypeptide consisting essentially of the EGF-like domain of human urokinase-type plasminogen activator, said method comprising:

providing a yeast host transformed with an expression vector, said vector comprising a transcriptional promoter operably linked to an oligonucleotide encoding a huPAR antagonist polypeptide consisting essentially of the EGF-like domain of human urokinase-type plasminogen activator or an active analog thereof;

culturing said yeast host under conditions which promote expression of said polypeptide; and

isolating said polypeptide.

2. The method of claim 1, wherein said oligonucleotide further encodes a signal leader polypeptide operatively connected to said huPAR antagonist polypeptide or analog, operative in said host cell to direct secretion of the expressed polypeptide.

3. The method of claim 2, wherein said signal leader comprises yeast α-factor leader.

4. The method of claim 3 wherein said yeast α-factor leader is *S. cerevisiae* α-factor leader.

5. The method of claim 1, wherein said host cell is *Saccharomyces cerevisiae*.

6. The method of claim 1, wherein said huPAR antagonist polypeptide consists essentially of $huPA_{1-48}$.

7. A huPAR antagonist polypeptide composition comprising a non-fucosylated polypeptide consisting essentially of the EGF-like domain of human urokinase-type plasminogen activator or an active analog thereof.

8. The composition of claim 7, wherein said non-fucosylated polypeptide consists essentially of $huPA_{1-48}$.

9. The composition of claim 7, further comprising a pharmaceutically acceptable excipient.

10. A method for treating a uPA-mediated disorder, said method comprising:

providing a composition comprising a non-fucosylated polypeptide consisting essentially of the EGF-like domain of human urokinase-type plasminogen activator or an active analog thereof; and

administering an effective amount of said composition to a patient having a uPA-mediated disorder.

11. The method of claim 10, wherein said polypeptide consists essentially of $huPA_{1-48}$.

12. The method of claim 10, wherein said uPA-mediated disorder is selected from the group consisting of metastasis, inappropriate angiogenesis, and chronic inflammation.

13. The method of claim 12, wherein said uPA-mediated disorder is selected from the group consisting of Kaposi's sarcoma, diabetic retinopathy, and rheumatoid arthritis.

14. The method of claim 10, wherein said composition is administered by instillation in the eye.

15. A method for pre-enriching a monovalent phage display mixture prior to screening for binding to a target, comprising:

(a) providing a mixture of monovalent display phage and non-displaying phage, wherein said monovalent display phage display both a candidate peptide and a common peptide, wherein said common peptide is identical for each monovalent display phage, and wherein said candidate peptide is different for different monovalent display phage; and

(b) separating all phage displaying a common peptide from phage not displaying a common peptide.

16. The method of claim 15, wherein said candidate peptide is $huPA_{1-48}$ or an active analog or active portion thereof.

17. The method of claim 15, wherein said common peptide comprises an antibody epitope.

18. The method of claim 17, wherein said epitope comprises Glu-Tyr-Met-Pro-Met-Glu.

19. The method of claim 15, further comprising:

(c) contacting said separated phage displaying said common peptide with said target; and

(d) separating phage which bind said target from phage which do not bind said target.

20. The method of claim 19, wherein said target comprises huPAR.

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
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(11) International Publication Number: WO 94/28145

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(30) Priority Data:
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(72) Inventors: ROSENBERG, Steven; 2323 Bywood Drive, Oakland, CA 94602 (US). STRATTON-THOMAS, Jennifer, R.; 166 14th Avenue, San Mateo, CA 94402 (US).

(74) Agents: GREEN, Grant, D. et al.; Chiron Corporation, Intellectual Property Dept. -R440, P.O. Box 8097, Emeryville, CA 94662-8097 (US).

(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, GE, HU, JP, KG, KP, KR, KZ, LK, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(88) Date of publication of the international search report:
19 October 1995 (19.10.95)

(54) Title: EXPRESSION OF UROKINASE PLASMINOGEN ACTIVATOR INHIBITORS

(57) Abstract

A method for preparing a urokinase-type plasminogen activator inhibitor by expressing $HuPA_{1-48}$ from yeast is disclosed.

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TJ	Tajikistan
TT	Trinidad and Tobago
UA	Ukraine
US	United States of America
UZ	Uzbekistan
VN	Viet Nam

INTERNATIONAL SEARCH REPORT

Interna al Application No
PCT/US 94/05669

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/58 C12N15/62 C12N9/72 A61K37/547 C12N7/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF BIOLOGICAL CHEMISTRY, vol.267, no.20, 15 July 1992, MD US pages 14151 - 14156 S. RABBANI ET AL 'Structural requirements for the growth factor activity of the aminoterminal domain of urokinase' cited in the application	7-12
Y	see the whole document especially 14154, left column, last line - right column, first paragraph --- -/--	1-6, 10-14

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

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"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

3

Date of the actual completion of the international search	Date of mailing of the international search report
24 May 1995	11. 09. 95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016	VAN DER SCHAAL C.A.

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Intern ial Application No
PCT/US 94/05669

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	S. KINGSMAN AND A. KINGSMAN 'Genetic engineering' , BLACKWELL SCIENTIFIC PUBLICATIONS , OXFORD see page 85, paragraph 3 - page 86, line 2 see page 445, last paragraph see page 86, last paragraph - page 87; figure 3.19	1-6
Y	JOURNAL OF BIOLOGICAL CHEMISTRY, vol.265, no.7, 5 March 1990, MD US pages 3793 - 3802 K. FREDERICK ET AL 'Glucose oxidase from Aspergillus niger' cited in the application see page 3794, right column, line 34 - line 40	2-6
Y	DATABASE WPI Section Ch, Week 2092, Derwent Publications Ltd., London, GB; Class B04, AN 92-167162 & WO,A,92 07083 (CANCER-FORSKNINGSFONDET) 30 April 1992 see abstract	10-14
A	JOURNAL OF BIOLOGICAL CHEMISTRY, vol.262, no.10, 5 April 1987, MD US pages 4437 - 4440 E. APPELLA ET AL 'The receptor-binding sequence of urokinase' see the whole document	
E	WO,A,94 22464 (THE GENERAL HOSPITAL CORPORATION) 13 October 1994 see the whole document	7-14
A	BIOCHEMISTRY, vol.30, 19 October 0 pages 10832 - 10838 H. LOWMAN ET AL 'Selecting high-affinity binding proteins by monovalent phage display' cited in the application see the whole document	

3

INTERNATIONAL SEARCH REPORT

.rnational application No.
PCT/US 94/05669

Box I Observations where certain claims were f und unsearchable (Continuation f item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 10-14
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 10-14 are directed to a method of treatment of the human body, the search has been carried out and based on the alleged effects of the composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

B x II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

- claims 1-14
- claims 15-20

See further information on the (2) enclosed sheets

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [X] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-14.

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

LACK OF UNITY OF INVENTION

1. Claims: 1-14
Method for producing non-fucosylated polypeptides consisting essentially of the EGF-like domain of human urokinase-type plasminogen activator the polypeptde and use thereof.

2. Claims: 15-20
Method of pre-enriching a monovalent phage display mixture

The first problem presented in the underlying application is to provide urokinase plasminogen activator inhibitors.

The solution to this problem is to provide a non-fucosylated polypeptide consisting essentially of the EGF-like domain of human urokinase-type plasminogen activator.

The second problem presented in the application is to improve the method for screening target binding peptides with the use of a monovalent phage display mixture described by H. Lowman Biochemistry 30(1991) 10832.

The solution to this second problem is to pre-enrich the monovalent phage display mixture prior to screening for binding to a target by providing a monovalent phage display mixture not only displaying the candidate peptides which whill be used to bind to the target, but also a common peptide which is common to all candidate peptide displaying phages and separating the common peptide displaying phages from wildtype phages.

The second solution can be used to screen for the EGF-like domain of human urokinase-type plasminogen activator or active analog or portion thereof (candidate peptides) which can bind to the human urokinase-type plasminogen receptor (target).

Other methods to screen and assay for binding of ligand and receptor has been described.
See f.i. page 2, line 18-page 3, line 21 and page 10, second paragraph of the application.

The ISA considers, that due to the fact that other methods exist to screen for receptor-binding ligands there is no technical relationship between an urokinase-type plasminogen activator receptor antagonist and the improvement of an assay to screen for receptor- binding compounds.

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INTERNATIONAL SEARCH REPORT

FURTHER INFORMATION CONTINUED FR M PCT/ISA/210

Consequently, there is no single inventive concept underlying the two claimed inventions and thus a lack of unity. The different inventions, not belonging to a common inventive concept, are formulated as the different subjects on the communication pursuant to Art 17 (3) (a) PCT.

MEANINGFUL SEARCH NOT POSSIBLE OR INCOMPLETE SEARCH

Remark: Although claims 10-14 are directed to a method of treatment of the human body the search has been carried out and based on the alleged effects of the composition.

INTERNATIONAL SEARCH REPORT

information on patent family members

Inter ıal Application No
PCT/US 94/05669

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9422464	13-10-94	AU-B- 6418794	24-10-94